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Desulfobacter vibrioformis sp. nov., a Sulfate Reducer from a Water-Oil Separation System

TORLEIV LIEN[1]* AND JANICHE BEEDER[2]

Department of Microbiology, University of Bergen, N-5020 Bergen,[1] and Hydro Research Centre, N-3901 Porsgrunn,[2] Norway

A mesophilic, gram-negative, vibrio-shaped, marine, acetate-oxidizing sulfate reducer (strain B54) was isolated from a water-oil separation system on a North Sea oil platform. The optimum conditions for growth were 33°C, pH 6.8 to 7.0, and concentrations of NaCl and $MgCl_2 \cdot 6H_2O$ of at least 1 and 0.3%, respectively. Of various organic acids tested, only acetate was used as an electron and carbon source. The presence of 2-oxoglutarate:dye oxidoreductase suggests acetate oxidation via an operative citric acid cycle. Even though growth of most *Desulfobacter* strains (including strain B54) did not occur on hydrogen, hydrogenase was detected at low activity. The growth yields were 4.6, 13.1, and 9.6 g of (dry weight) cells per mol of acetate oxidized with sulfate, sulfite, and thiosulfate, respectively, as electron acceptors. Strain B54 was able to fix dinitrogen. Desulforubidin and cytochromes of the *c* and *b* types were present. The G+C content of the DNA was 47 mol%. Strain B54 is most closely related to *Desulfobacter latus*, with a 16S rDNA sequence similarity of 98.1%. The DNA-DNA relatedness between them was 40.5%. On the basis of differences in genotypic, phenotypic, and immunological characteristics, we propose that strain B54 is a member of a new species, *D. vibrioformis*. It can be easily identified and distinguished from other *Desulfobacter* species by its large, vibrio-shaped cells.

In 1977, Widdel and Pfennig (44) isolated the first authenticated sulfate-reducing bacterium, *Desulfotomaculum acetoxidans*, which is able to oxidize acetate completely to carbon dioxide. Later, several new acetate-oxidizing sulfate reducers, both mesophilic and thermophilic, were described (4, 28, 43, 45). Most of these completely oxidizing sulfate reducers are nutritionally versatile, using the oxidative CO dehydrogenase pathway for acetate oxidation (36). The genus *Desulfobacter*, however, comprises nutritionally specialized sulfate reducers with acetate as their characteristic substrate, which they oxidize via a modified citric acid cycle (16, 25). The genus was established in 1981 by the description of the type species *Desulfobacter postgatei* (43). New *Desulfobacter* species were isolated in 1987, and the genus description was then emended to include H_2-utilizing, curved, and vibrio-shaped cells (40). Only a few *Desulfobacter* species, *D. postgatei*, *D. hydrogenophilus*, *D. latus*, and *D. curvatus*, have been validly described (40). Although other members of the *Desulfobacter* genus have been recently detected, e.g., by oligonucleotide probes, in various locations like bioreactors (33), oil field environments (7, 39), and photosynthetic biofilms (32), none of them has been isolated and characterized.

In this paper, we describe the general characteristics and phylogenetic relations of a marine, vibrio-shaped, sulfate-reducing bacterium belonging to the genus *Desulfobacter*. The strain which we describe B54^T [T = type strain]) was enriched and isolated with acetate and sulfate in a medium inoculated with water from the oil-water separation system on an oil production platform in the North Sea. Because of significant differences in genotypic, phenotypic, and immunological properties between strain B54^T and validly described members of the genus *Desulfobacter*, we propose that strain B54^T be placed in a new species, *D. vibrioformis*.

* Corresponding author. Mailing address: University of Bergen, IM, Jahnebakken 5, N-5020 Bergen, Norway. Phone: 47-55582669. Fax: 47-55589671. E-mail: torleiv.lien@im.uib.no.

(A preliminary account of some of these results has been reported previously [3, 23].)

MATERIALS AND METHODS

Sources of organisms. Strain B54^T was isolated from a water-oil separation system on the deck of the Statfjord B oil field platform in the Norwegian sector of the North Sea. The water samples were collected after the separation (flotation cell) of produced formation water from crude oil. The temperature at the sampling point was 38°C, and the pH was 7.95. *D. postgatei* (DSMZ 2034), *D. hydrogenophilus* (DSMZ 3380), *D. latus* (DSMZ 3381), and *D. curvatus* (DSMZ 3379) were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany.

Enrichment, isolation, and cultivation. Acetate-oxidizing sulfate reducers were enriched on a defined bicarbonate-buffered, sulfide-reduced marine medium (43) with 20 mM acetate as the substrate. The medium was dispensed into 50-ml screw-cap bottles. The bottles were inoculated with 5-ml water samples and incubated at 30°C in the dark. Enrichments with sulfide concentration and turbidity increases in three consecutive passages were regarded as positive. Pure cultures were isolated by serial dilution in anoxic agar medium (41). Medium L10, which was used for routine and large-scale cultivation and characterization studies, contained (per liter of distilled water) 6.0 g of $CH_3COONa \cdot 3H_2O$, 7.0 g of Na_2SO_4, 0.25 g of NH_4Cl, 1.0 g of $KH_2PO_4 \cdot 2H_2O$, 10 g of NaCl, 3.0 g of $MgCl_2 \cdot 6H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$, and 1 ml of trace element solution SL-10 (42). After autoclaving, the medium was cooled under a steady flow of oxygen-free nitrogen and then reduced by adding 4 ml of an anaerobically filter-sterilized 0.5 M $Na_2S \cdot 9H_2O$ solution per liter of medium. The pH was adjusted to 6.8 with 1 M Na_2CO_3. For dispensation of anoxic medium into (serum) bottles or culture tubes, the medium was prepared in a 3-liter Erlenmeyer flask equipped with a butyl rubber stopper and a device similar to that described by Widdel and Bak (41).

Morphology. Phase-contrast microscopy (Labophot; Nikon, Tokyo, Japan) was used for routine examinations and for photomicrography using the agar slide method (29). Platinum-palladium-shadowed cells were examined in an electron microscope (100 CX; JEOL, Tokyo, Japan).

Metabolism and physiology. Bellco tubes (no. 2047; 28 ml) containing 10 ml of L10 medium with 5% (vol/vol) inoculum were used for nutritional characterization and determination of temperature, pH, and salinity optima. Growth was determined by measuring the increase in optical density at 600 nm.

Sulfide was measured by the method of Cord-Ruwisch (11). Acetate was assayed by using a Varian high-performance liquid chromatograph (HPLC) equipped with a Perkin-Elmer LC-75 spectrophotometric detector and an Aminex HPX-87H column (Bio-Rad, Richmond, Calif.).

Pigments. Cytoplasmic and membrane fractions were made from 8 g (wet mass) of cells as described previously (34). The cytoplasmic fraction was dialyzed against 10 mM Tris-HCl buffer, pH 7.6, and proteins were separated on a DEAE-cellulose column as described by Samain et al. (35). The spectrum of the

various eluent fractions was recorded and checked for peaks characteristic of desulforubidin (22). Membrane-bound pigments were extracted from the membrane fraction with Triton X-100 (0.5%, vol/vol) as described previously (34). Cytochromes were identified in this extract and in the DEAE-cellulose eluate by spectroscopy of dithionite-reduced samples (37).

Serology. Polyclonal antiserum against strain B54^T was produced as described previously (10). Antigens were characterized by Western immunoblotting of sodium dodecyl sulfate (SDS)-soluble whole-cell extracts. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the whole-cell extracts was carried out as described by Laemmli (21), with 12.5% (wt/vol) and 4.5% (wt/vol) polyacrylamide in the separation and stacking gels, respectively. The PAGE was run at 190 V in a Mini Protean II Dual Slab Cell (Bio-Rad). After SDS-PAGE, the gels were stained with Coomassie brilliant blue (R-250; Serva). Unstained gels were immunoblotted as described by Burnette (8), by using a Mini Trans Blot Cell (Bio-Rad).

Enzyme assays. Preparation of cell extract was performed in an anoxic chamber or under flushing with oxygen-free gas. All assays were performed under strictly anaerobic conditions at 30°C. Carbon monoxide dehydrogenase and 2-oxoglutarate dehydrogenase were assayed as described previously (2). Hydrogenase activity was measured as H_2 consumption by monitoring benzyl viologen dye reduction at 555 nm in 100 mM Tris HCl buffer, pH 8.5, and as H_2 evolution from dithionite-reduced methyl viologen (17). Sulfite reductase was assayed with reduced methyl viologen as the electron donor (26). Nitrogenase activity was measured by using the acetylene reduction method (15). Protein was measured by the Bradford method (6).

DNA analyses. The G+C content of DNA (isolated as described by Beji et al. [5]) was determined after enzymatic digestion and HPLC separation of nucleosides (24). For the DNA-DNA hybridization studies, DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al. (9). The hybridization was carried out as described by De Ley et al. (12), with the modifications described by Huß et al. (18), using a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (19).

16S rDNA sequencing. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of PCR products were done as previously described (30, 31). Purified PCR products were sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. An Applied Biosystems 373A DNA sequencer was used for electrophoresis of the sequence reaction products. The 16S rDNA sequence was manually aligned against representative sequences of members of the delta subclass of *Proteobacteria*. Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (20). The least-squares distance of De Soete (13) was used to construct a phylogenetic dendrogram from distance matrices. The hybridization and sequence analyses were carried out at the Identification Service of the DSMZ by F. A. Rainey.

Nucleotide sequence accession number. The sequence of the 16S rDNA of strain B54^T has been deposited in the GenBank database under accession number U12254.

RESULTS

Enrichment and isolation. Enrichment cultures with acetate produced sulfide after 6 to 8 days of incubation at 30°C. The enrichments were subcultured, and a large, vibrio-shaped bacterium became dominant in several of the cultures after three consecutive passages. This strain, designated B54^T, was isolated in pure culture.

Morphology. The cells of strain B54^T were vibrio shaped, 1.9 to 2.3 by 4.5 to 8 μm in size, and nonmotile or motile with a single polar flagellum (Fig. 1). They were usually single or in pairs and sometimes arranged in chains but did not form aggregates. Electron micrographs of thin sections of cells revealed a gram-negative cell wall structure (not shown).

Pigments. The cytoplasmic fraction was subjected to anion-exchange chromatography on a DEAE-cellulose column. A reddish brown fraction eluted from the column was identified as desulforubidin by absorption maxima at 392, 545, and 580 nm and sulfite reductase activity. Dithionite-reduced spectra of a reddish fraction revealed absorption maxima at 416, 522, and 552 nm, indicating the presence of *c*-type cytochromes. Cytochromes of the *b* type (absorption maxima at 424, 526, and 558 nm) were extracted from the membrane fraction with 0.5% (vol/vol) Triton X-100.

Physiology. Temperature and pH optima and other growth characteristics are given in Table 1. Strain B54^T grew between

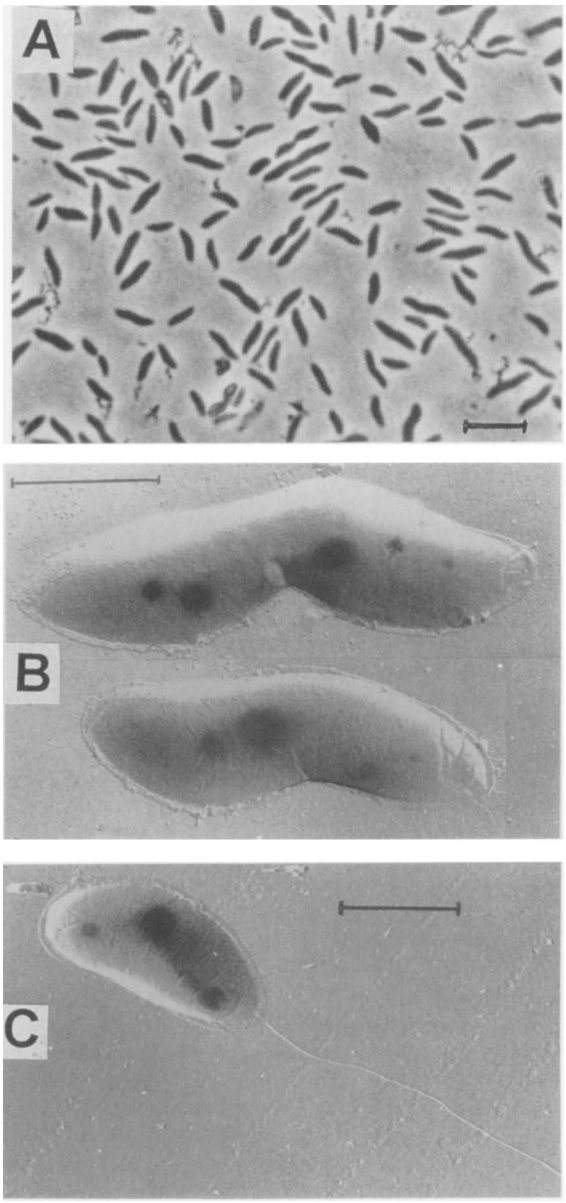

FIG. 1. Morphology of strain B54^T. (A) Phase-contrast micrograph of ceils. Bar, 10 μm. (B and C) Electron micrographs of platinum-palladium (diagonal)-shadowed cells, one of which (C) has a monopolar flagellum. Bars, 2 μm.

5 and 38°C. The best growth was obtained with phosphate-buffered L10 medium. The isolate required at least 0.3% (wt/vol) $Mg_2Cl \cdot 6H_2O$ and 1% (wt/vol) NaCl in the medium. Growth occurred equally well between 1 and 5% (wt/vol) NaCl. The isolate is able to fix dinitrogen, since it grew well in ammonia-free medium in the presence of N_2, and the cells from these cultures were positive for nitrogenase activity by the acetylene reduction test.

More than 30 different substrates were tested as carbon and/or energy sources and potential electron acceptors. The results showed that only acetate was used as a carbon and energy source with sulfate, sulfite, and thiosulfate as alternative electron acceptors. Acetate was oxidized to CO_2. The growth yields were 4.6, 13.1, and 9.5 g of cell dry weight per mol of oxidized acetate with sulfate, sulfite, and thiosulfate,

TABLE 1. Comparison of characteristics of *D. vibrioformis* and validly described *Desulfobacter* species

Characteristic	*D. vibrioformis* B54^T	*D. latus*[a]	*D. curvatus*[a]	*D. hydrogenophilus*[a]	*D. postgatei*[b]
Cell shape	Vibrioid	Oval, elongated	Vibrioid	Oval, elongated	Rod to ellipsoid
Cell sizes (μm)					
Width	1.9–2.5	1.6–2.4	0.5–1	1–1.3	1–1.5
Length	4.5–8	4–7	1.7–3.5	2–3	1.7–2.3
Motility	+	±	+	−	±
Temp optimum (°C)	33	29–32	28–31	29–32	32
pH optimum	6.8–7.0	7.0–7.3	6.8–7.2	6.6–7.0	7.3
Salinity range (% NaCl, $MgCl_2 \cdot 6H_2O$)	1–5, ≥0.3	2, 0.3	≥0.7, ≥0.13	2, 0.3	≥0.5, ≥0.1
Vitamin requirement(s)	None	Biotin, thiamine	Biotin	Biotin, *p*-aminobenzoate	Biotin, *p*-aminobenzoate
Shortest doubling time (h) on acetate	20	21	21	18	20
G+C content of DNA (mol%)	47	43.8	46.1	44.6	45.9
Electron donors					
Acetate	+	+	+	+	+
H_2	−	−	−	+	−
Ethanol	−	−	+	+	−
Pyruvate	−	−	+	+	−
Electron acceptors					
Sulfate	+	+	+	+	+
Sulfite	+	−	+	+	+
Thiosulfate	+	−	+	+	+

[a] Data from reference 40.
[b] Data from reference 43.

respectively. No dismutation of sulfite and thiosulfate was observed.

Cell extract of strain B54^T contained no carbon monoxide dehydrogenase activity but had a specific activity of 2-oxoglutarate dehydrogenase of 0.53 μmol · min^{-1} · mg of protein^{-1}, which indicates the presence of an operative citric acid cycle. The activities of hydrogenase in intact cells were 16 ± 2 and 7 ± 1 nmol · min^{-1} · mg of protein^{-1} for H_2 consumption and H_2 production, respectively. Hydrogen consumption activity was found in the whole cells and in the cytoplasmic and membrane fractions of all of the *Desulfobacter* species tested. The highest activity, 350 nmol · min^{-1} · mg of protein^{-1}, was assayed with intact cells of *D. curvatus*.

Whole-cell protein patterns. Although the *Desulfobacter* species have proteins in common, e.g., in the molecular mass range of 45 to 90 kDa, strain B54^T contained specific protein bands, e.g., at 18 to 20 and at 32 kDa, that differentiated it from the other *Desulfobacter* species (Fig. 2A).

Serological characterization. An antigen band with a molecular mass of 60 kDa was found in all of the *Desulfobacter* species tested (Fig. 2B). However, the immunoblotting demonstrated serological differences between the *Desulfobacter* species. Strain B54^T had several unique antigen bands, especially in the lower-molecular-mass range, which discriminate it from the other *Desulfobacter* species.

DNA base composition. The G+C content of DNA from strain B54^T was 47.4 ± 0.1 mol% as determined by the chemical method. The results also revealed that 1% of the adenines and 2% of the cytosines are methylated.

Phylogeny. 16S rDNA sequence analyses of strain B54^T showed it to group within the delta subclass of *Proteobacteria*. Figure 3 shows the relationship of strain B54^T to the main lines within the sulfate-reducing members of the delta subclass. It is clear from the phylogenetic dendrogram that strain B54^T is most closely related to the genus *Desulfobacter*. The similarity of the 16S rDNA sequence of strain B54^T to those of the validly described species of the genus *Desulfobacter* is in the range of 96.2 to 98.1%, and *D. latus* is the most closely related species.

DNA-DNA homology. The DNA-DNA homology of strain B54^T and *D. latus* was examined; the hybridization value was 40.5%.

DISCUSSION

North Sea formation water contains both acetate (1) and sulfate (4). Hence, mesophilic, acetate-oxidizing sulfate reducers are likely to be active in the part of this separation system with moderate and low temperature ranges. The finding of hydrogenase activity in all of the *Desulfobacter* species tested was unexpected, since only *D. hydrogenophilus* are able to grow with hydrogen (40). It may be that under certain growth conditions, the other *Desulfobacter* spp. are also able to utilize hydrogen. Furthermore, the observed low hydrogenase activity for hydrogen production may be connected to the modified citric acid cycle in *Desulfobacter* spp. with ferredoxin as an electron acceptor (25).

Strain B54^T was identified as a member of the genus *Desulfobacter* on the basis of phenotypic characteristics, e.g., the ability to grow solely on acetate as a substrate in a dissimilatory sulfate reduction to sulfide. This identification was confirmed by 16S rDNA sequence analyses, which showed that strain B54^T is most closely related to *D. latus* (level of sequence similarity, 98.1%). However, 16S rDNA sequence analyses are unable to separate closely related species with similarity levels of 97% or higher. Their relationships are best determined by phenetic and genetic similarities (14, 27, 38). Strain B54^T differs phenotypically, serologically, and genetically from the other validly described *Desulfobacter* species. First, strain B54^T has a distinct morphology, with large vibrio-shaped (curved)

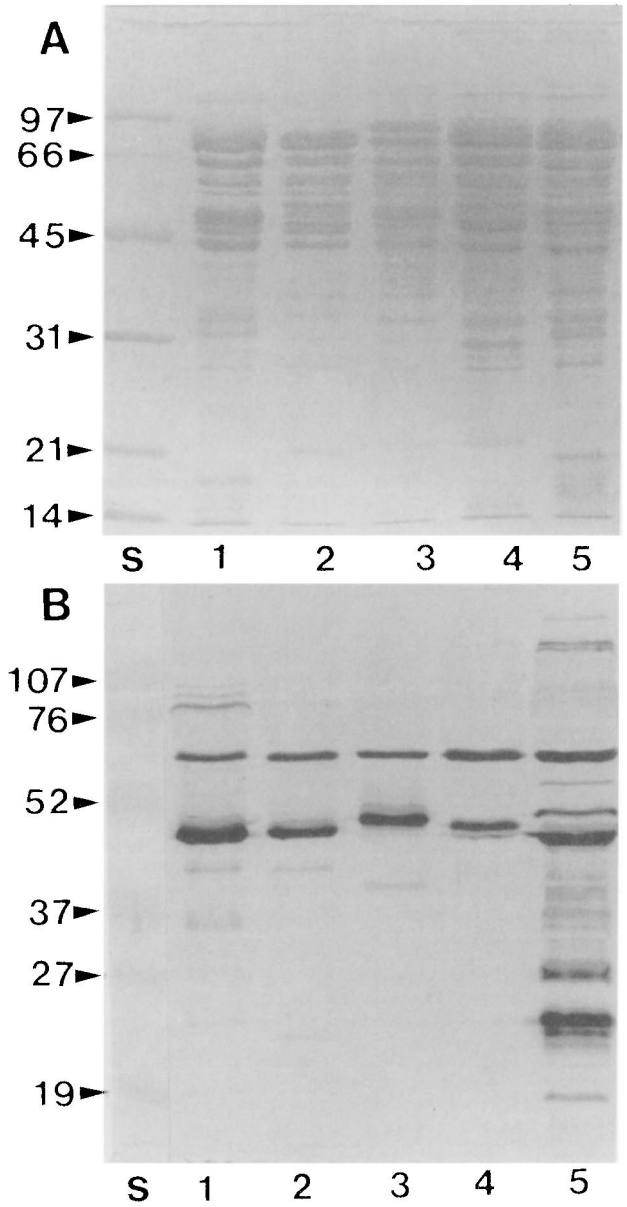

FIG. 2. Protein patterns (A) and immunoblotting with anti-B54^T antibody (B) after SDS-PAGE of SDS-soluble whole-cell extracts of *D. postgatei* (lanes 1), *D. curvatus* (lanes 2), *D. hydrogenophilus* (lanes 3), *D. latus* (lanes 4), and strain B54^T (lanes 5). Molecular masses of standards (lanes S) are indicated (in kilodaltons) on the left.

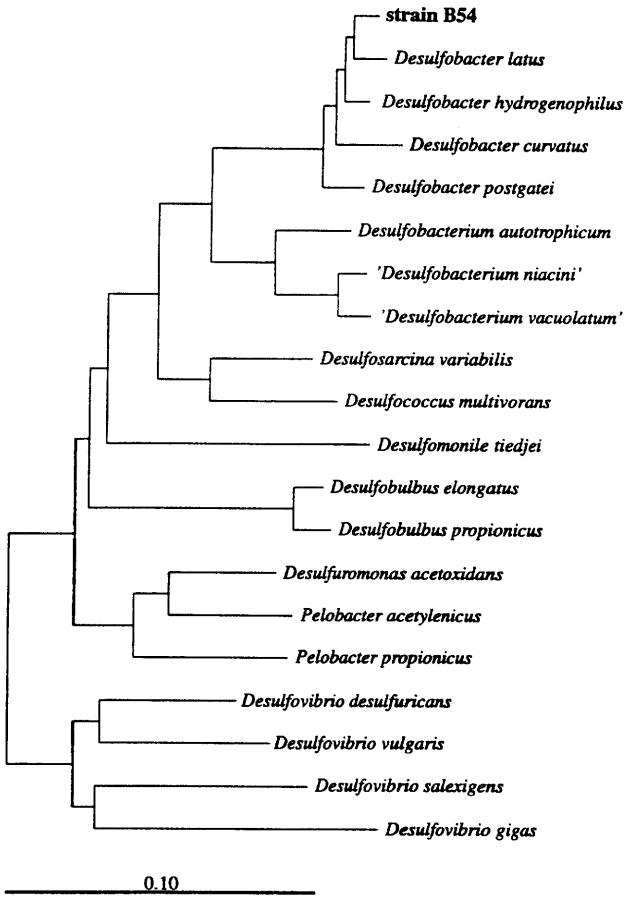

FIG. 3. Phylogenetic dendrogram based on 16S rDNA sequence comparison, indicating the positions of strain B54^T within the radiation of the sulfate-reducing bacteria of the delta subclass of *Proteobacteria*. Bar, 10 nucleotide changes per 100 nucleotides.

cells that are frequently paired. Second, strain B54^T has no vitamin requirement. In contrast to *D. latus*, it utilizes sulfite and thiosulfate as alternative electron acceptors (Table 1). So does the marine, vibrio-shaped species *D. curvatus*, but it grows best with ethanol (40). Third, the protein profile and immunological properties of B54^T are different from those of the other *Desulfobacter* species. Fourth, strain B54^T has a slightly higher G+C content than the other *Desulfobacter* species. Finally, the level of DNA-DNA hybridization of 40.5% between strain B54^T and *D. latus* is, according to the current criterion (38), too low for these organisms to be considered members of the same species. Therefore, it appears necessary to establish a new species, for which we propose the name *Desulfobacter vibrioformis.*

Description of *Desulfobacter vibrioformis* sp. nov. *Desulfobacter vibrioformis* (vi.bri.o.for'mis. L. v. *vibrio*, vibrate; M. L. n. *vibrio*, that which vibrates, a generic name; L. adj. suffix *-formis*, -like, of the shape of; *vibrioformis*, vibrio shaped). Vibrio-shaped cells, 1.9 to 2.3 by 4.5 to 8 μm, usually single or in pairs and sometimes in chains. Do not form aggregates. Nonmotile or motile with a single polar flagellum. Reduces sulfate, sulfite, and thiosulfate. Acetate is the only electron donor and carbon source used. Able to fix N_2. No vitamins but at least 1% NaCl and 0.3% $MgCl \cdot 6H_2O$ are required. Growth occurs equally well between 1 and 5% NaCl in the medium. The temperature range for growth is 5 to 38°C; the optimum is 33°C. The optimum pH is 6.8 to 7.0. Desulforubidin, cytochromes *c* and *b* are present. The DNA base composition is 47 mol% G+C (as determined by HPLC). Isolated from production water from an oil separation system on a North Sea oil production platform. The type strain is B54^T (= DSMZ 8776).

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